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Swelling and Contraction of Corn Mitochondria[1]

C. D. Stoner[2] and J. B. Hanson
Department of Agronomy, University of Illinois, Urbana, Illinois

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Summary. A survey has been made of the properties of corn mitochondria in swelling and contraction. The mitochondria swell spontaneously in KCl but not in sucrose. Aged mitochondria will swell rapidly in sucrose if treated with citrate or EDTA. Swelling does not impair oxidative phosphorylation if bovine serum albumin is present.

Contraction can be maintained or initiated with ATP + Mg or an oxidizable substrate, contraction being more rapid with the substrate. Magnesium is not required for substrate powered contraction. Contraction powered by ATP is accompanied by the release of phosphate. Oligomycin inhibits both ATP-powered contraction and the release of phosphate. However, it does not affect substrate-powered contraction. Substrate powered contraction is inhibited by electron-transport inhibitors. The uncoupler, carbonyl cyanide *m*-chlorophenyl hydrazone, accelerates swelling and inhibits both ATP- and substrate-powered contraction. However, the concentrations required are well in excess of those required to produce uncoupling and to accelerate adenosine triphosphatase; the concentrations required inhibit respiration in a phosphorylating medium.

Phosphate is a very effective inhibitor of succinate-powered contraction. Neither oligomycin nor Mg affects the phosphate inhibition. Phosphate is less inhibitory with the ATP-powered contraction.

The results are discussed in terms of a hypothesis that contraction is associated with a nonphosphorylated high energy intermediate of oxidative phosphorylation.

Swelling and contraction of animal mitochondria have been extensively investigated, and are the subject of 2 excellent reviews (2, 15). It is established that within the limits imposed by their extensibility and compressibility mitochondria behave as osmometers, and that they are relatively permeable to salts such as KCl, but much less so to sucrose and other polyhydroxy compounds. Osmotic adjustment is obtained within a few seconds on changing osmolarity, but there is a slow swelling (spontaneous swelling) which can be accelerated by a wide variety of swelling agents, and which is largely reversible by ATP or phosphorylating respiration. The swelling is somehow linked to electron transfer and inhibitors of respiration prevent swelling. The contractile mechanism is thought to reside in the membrane, and is linked in some fashion to the intermediate system of oxidative phosphorylation. Swelling seems to result from relaxation of the contractile mechanism, possibly a mechanoprotein (15), which is reestablished by an ATP-consuming process with a concomitant extrusion of water.

Very little of this brief summary can be applied to plant mitochondria. Honda and Muenster (10) studied the relationship between swelling and succinate oxidation of lupine mitochondria, using both packed volume and light-scattering measurements. While packed volume measurements indicated that succinate sometimes prevented osmotically induced swelling and induced the mitochondria to contract, parallel optical studies indicated that succinate induced the mitochondria to swell. Osmotic swelling brought about either an activation or inhibition of succinate oxidation depending on the concentration of succinate used. These studies pointed out the need for a more complete characterization of swelling and contraction phenomena in plant mitochondria.

Lyons and Pratt (18) have studied the effect of ethylene on swelling and contraction of rat liver and cauliflower mitochondria. Both types swelled spontaneously in KCl and contracted upon addition of ATP and Mg. Swelling did not occur in sucrose. In related work, Lyons, Wheaton and Pratt (19) investigated the extent of mitochondrial swelling as related to chilling resistance of various plant tissues.

Chloroplasts undergo reversible light-scattering changes upon illumination, and Packer et al. (24) suggest that intermediates of phosphorylation in both mitochondria and chloroplast membranes are capable of bringing about structural changes in the membranes. Dilley (4) has recently related chloroplast

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[2] Present address: Heart Biochemistry Laboratory, University Hospital, Ohio State University, Columbus, Ohio.

shrinkage to a light-dependent potassium efflux.

As part of a study on membrane properties of corn mitochondria we thought it would be desirable to have information on reversible swelling, and the experiments are reported here. Corn mitochondria appear to differ from most animal preparations in that they show rapid spontaneous swelling, and will readily contract on addition of substrate alone. However, we suspect that these distinctions may result from a greater permeability of corn mitochondria, rather than from differences in the basic mechanism concerned.

Materials and Methods

Isolation of Mitochondria. Corn seedlings (*Zea mays*, WF9 × M14) were grown in the dark at 28 to 30° on paper towels saturated with 0.1 mM $CaCl_2$. About 150 g of 3 and one-half day shoots were ground in a cold mortar with 300 ml of 0.4 M sucrose + 5 mM EDTA + 0.1 M Tris + 0.05 M maleate (pH 7.5). The mitochondria were sedimented at 12,000 × *g* for 10 minutes after clearing the homogenate at 2000 × *g* for 10 minutes, and were washed once in the grinding medium and once in 0.4 M sucrose + 5 mM EDTA. Final suspension was in 0.4 M sucrose.

Oxidative Phosphorylation. Respiration was measured with the Warburg respirometer at 30°. The vessels contained in 2.5 ml volume: 450 μmoles sucrose, 125 μmoles glucose, 40 μmoles each of pyruvate and malate, 50 μmoles KH_2PO_4, 2.5 μmoles $MgSO_4$, 2.5 μmoles ADP, 0.6 μmole, NAD, 0.4 μmole thiamine pyrophosphate, 0.1 μmole CoA, and 25 KM units hexokinase, adjusted to pH 7.5. Respiration was generally followed for 20 minutes after 10 minutes equilibration.

Swelling and Contraction. Changes in light scattering were followed by changes in OD at 520 mμ with a Coleman Model 11 spectrophotometer. The stepwise procedure consisted of: 1) placing 4.0 ml of 0.25 M KCl + 0.025 M Tris-HCl buffer, pH 7.5, in matched tubes; 2) bringing the volume to 4.95 ml with water or experimental additives; 3) equilibrating the solutions at 28° in a water bath; 4) injecting 0.05 ml of mitochondrial suspension containing 0.12 to 0.15 mg N; 5) mixing by shaking and 6) following changes in OD. In experiments where pH was varied, Tris-maleate buffer was used. In a few early experiments, 0.1 ml of mitochondria was added to 4.9 ml of medium. Between measurements the tubes were held in the water bath. Contraction was initiated by adding the indicated additives in a small volume (0.02-0.05 ml), and corrections were made for the small change in OD due to dilution. Ethanol was used as solvent for some reagents, but a series of experiments showed the small amount used to be without significant effect on swelling or contraction; ethanol blanks were used in control treatments.

For gravimetric determinations parallel treatments were used with larger aliquots of mitochondria (ca. 0.9 mg N). At the end of the swelling-contraction period, the solutions were centrifuged at 25,000 × *g* for 10 minutes, the tube carefully drained and the bottom of the lusteroid tube containing the pellet was cut away. Fresh, oven dry and tare weights were obtained. Calculations were based on the change in water content of the pellet upon swelling and contraction, and no effort was made to estimate occluded salts and water.

Electron Microscopy. Phosphate buffer (0.01 M, pH 7.5) was used in these swelling-contraction studies because Tris reacts with the osmium fixative. At the end of the swelling-contraction treatment, the suspensions were chilled on ice, and 4 % OsO_4 in 0.2 M KCl was added with vigorous stirring to a final concentration of 0.5 % OsO_4. An equivalent aliquot of mitochondria held cold from the beginning in 0.4 M sucrose + 0.01 M potassium phosphate (pH 7.5) was similarly fixed. The mitochondria were collected as a thin pellet (ca. 0.2 mm thick) in flat-bottomed test tubes in a swinging bucket rotor at 1500 × *g* for 15 minutes. The clear supernatant was decanted and fresh fixative (1 % OsO_4 in KCl or sucrose plus buffer as above) added. After 30 minutes on ice, the fixative was replaced with half-strength buffered KCl or sucrose for 15 minutes. The pellets were dehydrated and embedded in methyacrylate with the use of 0.01 % uranyl nitrate (37) and a nitrogen atmosphere (23) to help prevent uneven polymerization. Sections were cut on an LKB microtome, stained for 1 hour with 4 % uranyl acetate, and viewed with a Hitachi HS-6 electron microscope.

Adenosine Triphosphatase Determinations. The basic medium was the same as that used for swelling-contraction studies. Final concentrations in 5 ml were 0.2 M KCl, 0.02 M Tris-HCl (pH 7.5), 5 mM ATP, 1 mM $MgCl_2$, 10 mM sucrose (added with the mitochondria), and 0.15 to 0.20 mg mitochondrial N. Media were equilibrated and held at 28°. Determinations of P_i (6) were made on 2 ml aliquots at 1 minute and at 31 minutes after adding the mitochondria. The reaction was stopped with ice cold 5 % trichloroacetic acid (final concentration) and the mitochondrial precipitate cleared by centrifugation.

Results

Spontaneous Swelling and Its Reversal with ATP. Preliminary work established that corn mitochondria do not swell appreciably in sucrose, but will swell spontaneously when transferred to isoosmolar KCl. Lyons et al. (19) made similar findings with a variety of plant mitochondria. When the same osmolarity is maintained by varying proportions of sucrose and KCl, the degree of spontaneous swelling increases with the concentration of KCl (fig 1). The initial OD also rises with increased proportion of KCl, which is probably due to the decreasing refractive index of the medium (33).

Does the decrease in OD when mitochondria are injected into KCl solutions actually represent swelling? The literature on swelling of animal mitochon

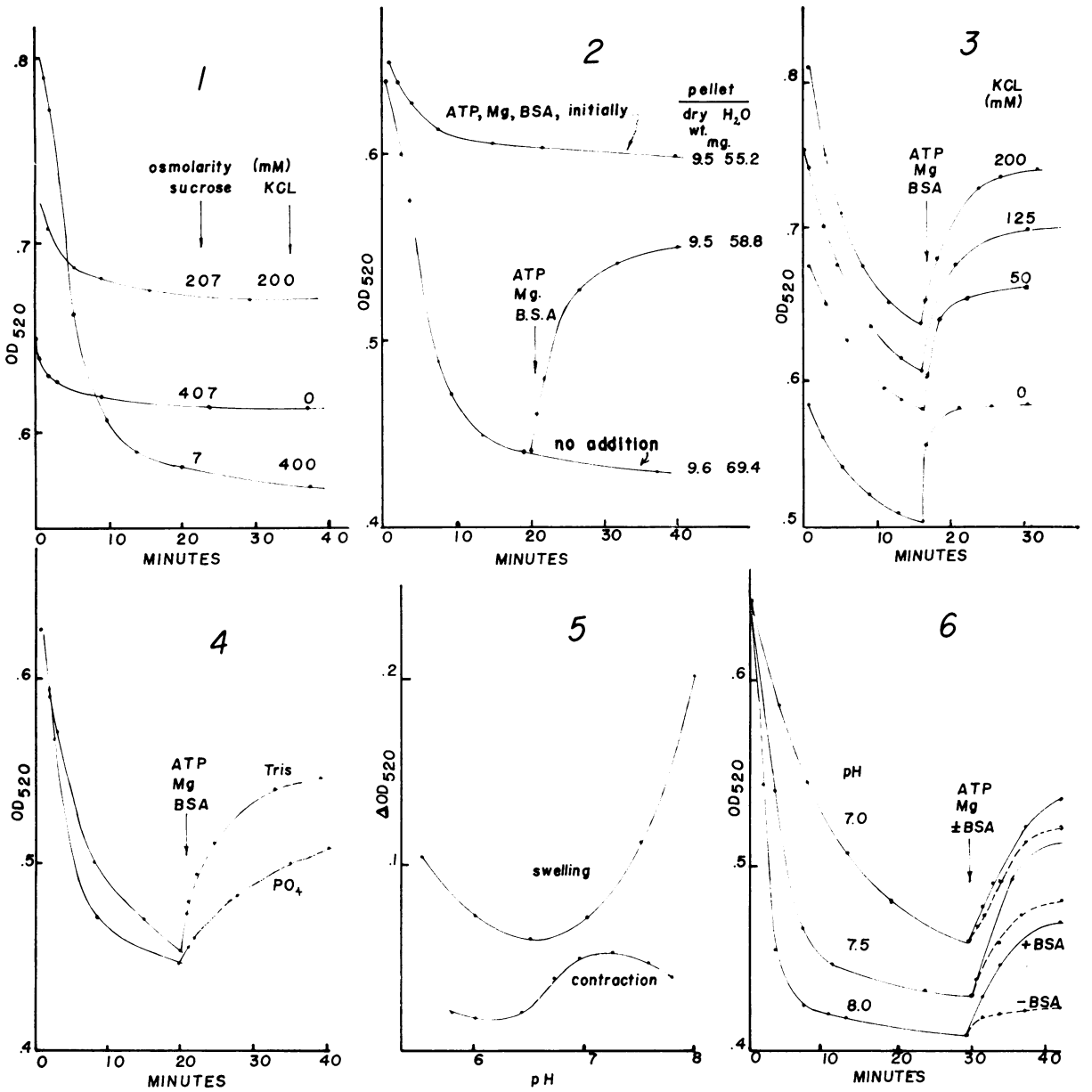


FIG. 1. The promotion of swelling in corn shoot mitochondria by KCl as indicated by loss of OD. Basic medium was 0.02 M Tris, pH 7.5 with KCl and sucrose as indicated.

FIG. 2. Relationship between OD change and water content of mitochondrial pellet. Basic medium was 0.2 M KCl + 0.02 M Tris, pH 7.5. Contraction initiated or maintained with 3 mM ATP, 2 mM $MgCl_2$ and 1 mg/ml BSA.

FIG. 3. Mitochondrial swelling and contraction in varying concentrations of KCl. Media were buffered with 0.02 M Tris-HCl, pH 7.5. Contraction initiated with 5 mM ATP, 3 mM $MgCl_2$ and 2 mg/ml BSA.

FIG. 4. Comparison of swelling and contraction in 0.02 M Tris or potassium phosphate buffer, pH 7.5. Contraction initiated with 1 mM ATP, 1 mM $MgCl_2$ and 1mg/ml BSA.

FIG. 5. Swelling and contraction as a function of pH. The curves present changes in OD 5 minutes after initiation of swelling or contraction in separate experiments. Swelling was carried out in 0.2 M KCl in 0.02 M Tris-maleate buffer. For contraction, mitochondria were allowed to swell in 0.2 M KCl + 0.005 M Tris-HCl, pH 7.5. At 20 minutes 0.05 ml of 1 M Tris-malate buffer of varying pH was added, and contraction was initiated by 3 mM ATP, 2 mM $MgCl_2$ and 1 mg/ml BSA. The pH readings are those taken at the end of the contraction period.

FIG. 6. The effect of high pH in accelerating swelling and increasing the BSA requirement for contraction. Contraction initiated with 1 mM ATP, 1 mM $MgCl_2$ with (solid line) and without (dashed line) 1 mg/ml BSA.

dria suggests 2 major tests: Is there an increase in water content of the mitochondria? Will ATP + Mg reverse the decrease in OD, extruding water? An experiment summarizing the results obtained from investigating these questions is shown in figure 2. Bovine serum albumin[3] was included with the additions of ATP and Mg to bind any fatty acids (U-factor) which might be released during the swelling period (17, 38).

The addition of ATP + Mg prevented most of the decrease in OD when added initially and largely reversed the decrease when added to swollen mitochondria. There was a close correspondence between the final OD of the suspensions and the water content of mitochondria recovered from them. If the mitochondria maintained in ATP are taken to represent 100 % contraction, and those fully swollen to represent zero contraction, then the addition of ATP to swollen mitochondria gave 72 % contraction by photometric measurement and 75 % by gravimetric. It was concluded that light scattering measurements give a good indication of the level of water uptake and extrusion.

Figure 3 shows the responses when the mitochondria suspended in 0.4 M sucrose were injected into cuvettes with KCl concentrations varying from isoosmolar (0.2 M) to buffer alone. As would be predicted from the work of Tedeschi and Harris (32, 33), there was a rapid osmotic adjustment prior to the time the first reading was made (30 sec), which resulted in lower initial ODs with lower KCl concentrations. Following this adjustment, an additional slow spontaneous swelling ensued, being greater in extent with increasing KCl.

Very likely the penetration of salt is responsible for the greater swelling which occurred in the system with isoosmolar KCl (3, 31). Where only buffer (0.02 M Tris) was present, however, the initial osmotic adjustment (swelling) was large and the additional subsequent swelling probably represents further stretching of mitochondrial membranes which approach their limits of extensibility. The relatively low level of ATP-induced reversal of the total swelling (initial rapid osmotic adjustment plus subsequent slow adjustment) in the hypotonic media is probably a reflection of damage to the contractile mechanism as a result of the greater extent of swelling, and the establishment of equilibrium at a low external osmotic pressure.

Tedeschi and Harris (33) point out that most of the difficulties arising from refractive index changes can be avoided by using media of nearly constant composition. It was decided to use 0.2 M KCl (approximately isoosmotic with the 0.4 M sucrose used in isolation) in all subsequent experiments. The rapid osmotic adjustments would be avoided, and although complete expulsion of water gained during spontaneous swelling could not be expected, there was no reason to think that the functioning of the contractile mechanism was impaired.

In view of the fact that the contractile mechanism is thought to be closely associated with the high energy intermediate system of oxidative phosphorylation (15, 24, 26), it was important to know how swelling in KCl affected oxidative phosphorylation. As shown in table 1, a pretreatment in KCl which would allow spontaneous swelling did not uncouple the mitochondria. However, compared with the appropriate control (addition of ATP + Mg + BSA to maintain contraction), there was some decline in substrate oxidation. The higher level of oxidation cannot be attributed to maintenance of contraction, for BSA alone was equally effective, and it was established in separate experiments that BSA did not prevent spontaneous swelling or initiate contraction. The important point is that the spontaneous swelling leaves the coupling mechanism functional.

In other experiments not detailed here, mitochondria were transferred to sucrose of differing osmolarity and changes in OD and water content were measured. Except at very low sucrose concentrations (0.05 M or less), there was no significant spontaneous swelling following the initial rapid osmotic adjustment. Between 0.5 M and 0.005 M sucrose, the water content of the mitochondrial pellets increased 25 %.

Swelling and contraction in phosphate and Tris buffers were compared (fig 4). Phosphate (20 mM) accelerated the rate of swelling and inhibited the rate of contraction. In another experiment, BSA (1 mg/ml) was found to slightly retard the swelling rate. Ethanol (1 %) did not affect swelling, but slightly inhibited the ATP-induced contraction rate.

Figure 5 shows levels of swelling and contraction at varying pH. Minimal swelling rates occur at pH 6.5, and maximal contraction rates at pH 7.2 to 7.4. The accelerated swelling above pH 6.5 might be related to release of fatty acids (U-factor), as the dependence upon BSA for contraction increases with pH (fig 6).

Table I. *Effect of Pretreatment in Tris-Buffered KCl on Subsequent Oxidative Phosphorylation*

Washed mitochondrial pellets were suspended in the swelling medium (0.2 M KCl + 0.02 M Tris, pH 7.5) containing 5 mM ATP, 3 mM $MgCl_2$ and 2 mg/ml BSA as indicated. After 20 minutes preincubation at 30° aliquots were transferred to Warburg vessels and oxidative phosphorylation measured with the pyruvate-malate medium. The sucrose was omitted, and the vessel increased by 40 mM KCl and 4 mM Tris added with the mitochondria.

Preincubation additives	Qo_2 (N)	P/O
None	790	2.3
ATP, Mg, BSA	1190	2.4
ATP, Mg	1180	2.5
BSA	1120	2.4

[3] Abbreviations used: BSA, bovine serum albumin. CCP, carbonyl cyanide *m*-chlorophenyl hydrazone.

For further work, it was decided to continue using pH 7.5 where both swelling and contraction would be rapid. Bovine serum albumin was routinely added at the time contraction was initiated.

Electron Microscopy. Mitochondria from an experiment like that of figure 2 were examined with the electron microscope. We were not skilled enough in electron microscopy to follow structural changes in fine detail, but gross morphological changes in the inner membrane were readily apparent (fig 7). Mitochondria held in sucrose or ATP show dense cristae or involutions of the inner membrane. The mitochondria in 0.4 M sucrose appear to have an inner membrane which is partially plasmolyzed, while the less dense outer membrane seems unaffected (fig 7a). This observation is in accord with suggestions that the outer membrane is quite permeable (1, 30, 36). The mitochondria held contracted in KCl by ATP show little or none of the plasmolysis but the inner membrane is very electron-dense, and the cristae are large (fig 7b).

Upon swelling in KCl, the large cristae seem to be withdrawn into narrow tubules, which present circular profiles in cross section (fig 7c). In median sections of the mitochondria, a peripheral location for these tubules is shown. When the swollen mitochondria are contracted by ATP (fig 7d), the large, dense cristae reappear in most mitochondria, but there seem to be fewer mitochondria where the cristae occupy the entire volume delineated by the outer membrane.

Characteristics of ATP-Induced Contraction. The optimal concentration of ATP for contraction was 1 to 3 mM. Above 3 mM ATP, the final level of contraction was somewhat reduced. The ATP-induced contraction was accompanied by a release of inorganic phosphate (fig 8). The contracting mitochondria thus exhibited an adenosine triphosphatase activity. Over the first 4 minutes when contraction was most rapid, the rate averaged 39 μmoles Pi/mg N per hour. Subsequently, the rate slowed to 26 μmoles/mg N per hour, and was maintained at this level throughout the incubation, even after contraction was essentially complete.

It was found that ADP (with Mg and BSA) would gradually induce contraction after a delay of about a minute. However, when hexokinase and glucose were added with the ADP, there was no contraction. This result was interpreted to mean that adenylate kinase, known to be associated with plant mitochondria (7), produced sufficient ATP to activate contraction, but that in the presence of a hexokinase-glucose trap the ATP did not accumulate to adequate levels. The addition of AMP (with Mg and BSA) did not effect contraction; neither did Mg nor BSA, alone or in combination.

Oligomycin strongly inhibited ATP-induced contraction (a value is included with table III), but oligomycin had no effect on swelling rates. This result is similar to that obtained by Packer et al. (24) with heart mitochondria. When ATP was added initially to maintain the contracted state, oligomycin initiated swelling and inhibited the release of Pi (fig 9). Oligomycin is thought to interfere at a terminal step with the reversible transfer of Pi between ATP and a phosphorylated intermediate of oxidative phosphorylation (14). Hence, it can be deduced that maintenance of contraction in KCl requires a continuous input of energy into the high energy intermediate system via a process which releases Pi from ATP.

The effect of oligomycin was of sufficient importance that we made observations on the effectiveness of the compound as an inhibitor of oxidative phosphorylation and adenosine triphosphatase (table II). At about 0.04 μg/ml (1.3 mμmole oligomycin/mg mitochondrial N), oxidative phosphorylation was practically eliminated and O_2 consumption was reduced by half. Beyond this point additional oligomycin had no effect, suggesting that all of the terminal phosphate transfer system was already blocked. It is tempting to deduce that only half of the electron transfer is coupled to phosphorylation, but this would be inconsistent with the good P/O ratios obtained in the controls. There is an alternative possibility: some coupled respiration might be shifted to an oligomycin insensitive process such as the salt uptake known to occur in these mitochondria (9) or to maintenance of contraction (see later).

The adenosine triphosphatase study was in KCl under conditions similar to those of figure 9, and thus not directly comparable with that of oxidative phosphorylation with its complex medium containing sucrose, substrates and cofactors. About 0.05 μg oligomycin/ml (3.9 mμmoles/mg N) were required before the mitochondria showed evidence of saturation (table II). However, the concentrations of oligomycin needed for half maximal inhibition are similar; 0.81 and 0.90 mμmoles/mg N for oxidative phosphorylation and adenosine triphosphatase. About 20 to 25 % of the adenosine triphosphatase activity measured in the contraction experiments is oligomycin insensitive.

The contraction maintained by the ATP-hydrolyzing system can be uncoupled by 10^{-5} M CCP (fig

Table II. *Inhibition of Oxidative Phosphorylation and Adenosine Triphosphatase by Oligomycin*

All treatments contained 2 % ethanol.

Oligomycin μg/ml	Qo_2 (N)	P/O	Adenosine triphosphatase μmoles Pi/hr per mg N
None	2150	2.6	44
0 01			29
0 02	1840	2.3	21
0 04	1080	0.4	
0.05			14
0.10	1080	0.2	12
0.20	1130	0.1	12
0.40	1150	0.2	
1.00			10
2.00	1290	0.1	

10) with no significant change in Pi released. In other work it was found that CCP added with oligomycin produced no change over oligomycin alone (i.e., as in fig 9), indicating that uncoupling is subsequent to the oligomycin-sensitive site in ATP-powered contraction.

The effectiveness of CCP on oxidative phosphorylation and adenosine triphosphatase is shown in

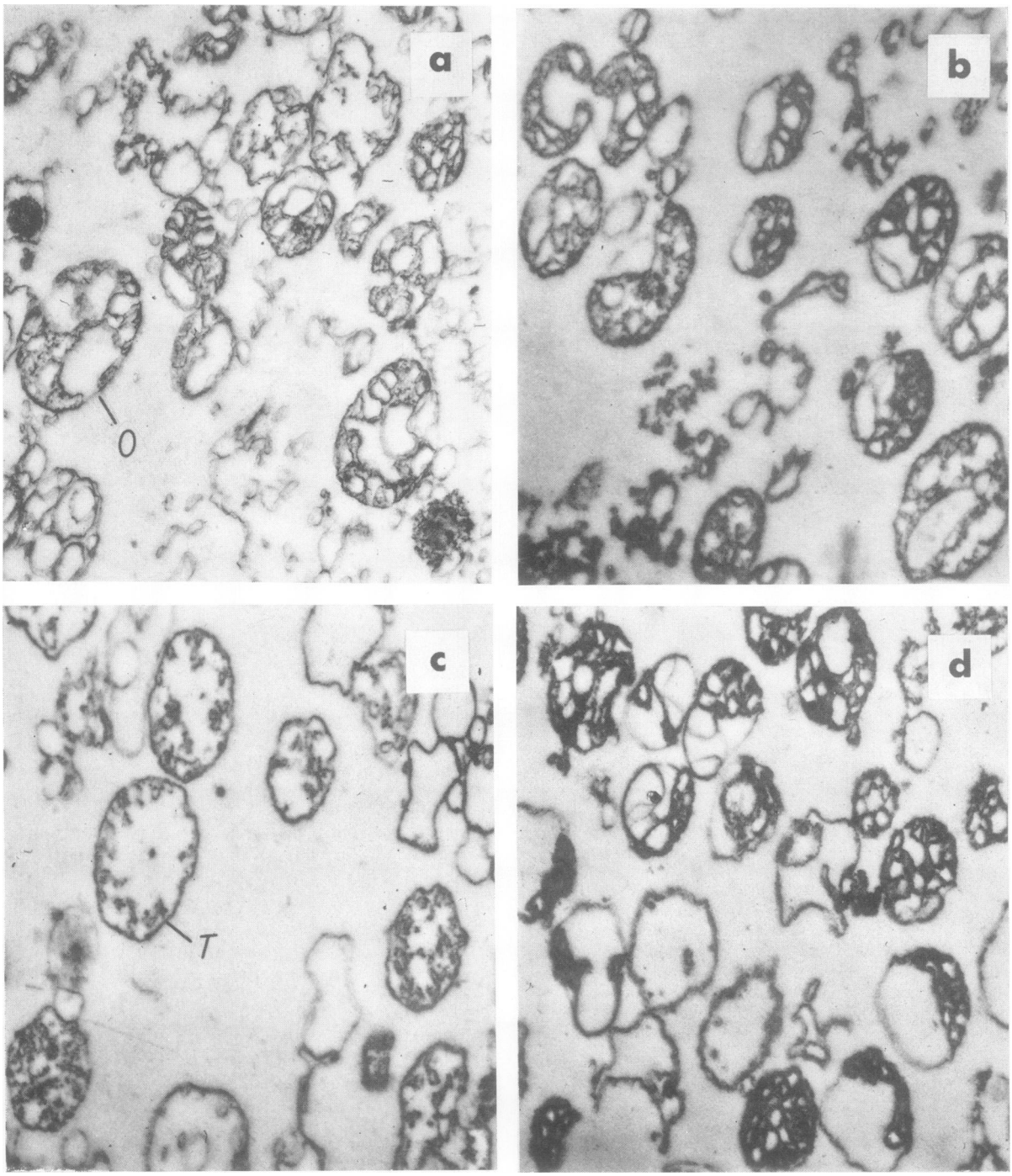


FIG. 7. Electron micrographs of mitochondria in various stages of swelling and contraction. Treatments as in figure 2: a) Initial state in 0.4 M sucrose; b) Maintained in contracted state; c) Swollen state; d) Contracted with ATP after swelling. Magnification × 30,000. *O*, Outer membrane from which the inner membrane has been withdrawn by plasmolysis leaving a large intermembrane space. *T*, Median section of a swollen mitochondrion showing peripheral tubular cristae.

figure 11. The oxidation rate of pyruvate-malate is maintained until phosphorylation is almost completely uncoupled. The adenosine triphosphatase is promoted by concentrations of CCP which uncouple. With CCP concentrations in excess of 10^{-7} both adenosine triphosphatase and electron transfer decline to lower levels, but are not eliminated. No stimulation of adenosine triphosphatase occurs if 0.5 M sucrose is added to the KCl + Tris medium. This protective action of sucrose may account for the failure of some investigators to find a DNP-stimulated adenosine triphosphatase (27, 35). We find a DNP-stimulated adenosine triphosphatase in KCl (29).

The effect of CCP on swelling rates is shown in figure 12. At high concentrations (10^{-5} M), the uncoupler accelerates swelling, but does not alter the final swollen volume. The same is true for DNP (29). Since the concentrations required are above those needed for uncoupling, it is dubious that the acceleration of swelling is directly related to uncoupling.

Figure 13 depicts the effectiveness of CCP as a function of concentration on contraction with and without BSA. The BSA must bind some CCP for its effectiveness is sharply reduced in the presence of BSA. Concentrations of CCP which uncouple oxidative phosphorylation (making due allowance for the BSA binding) and increase adenosine triphosphatase begin to inhibit contraction, but there is an inexplicable reversal of this trend at those concentrations where respiration and adenosine triphosphatase begin to decline (cf. fig 11). Still higher concentrations are inhibitory.

The Mg requirement for ATP-induced contraction is illustrated in figure 14. The endogenous Mg is apparently adequate to permit some contraction, but addition of Mg is promotive. Although not shown, the addition of Mg alone is without effect.

Substrate-Induced Contraction. Figure 14 also shows a surprising discovery made at this time. The general requirement for substrate oxidation to produce large amplitude swelling in animal mitochondria (2, 15) suggested that the addition of succinate might produce a further increment of swelling in corn mitochondria. The opposite effect was realized: the mitochondria contracted rapidly (half-time of about 30 seconds versus 3 to 4 minutes for ATP). There was no apparent requirement for Mg, and further work fully verified that exogenous Mg was neither needed nor promotive.

In contrast to the lack of response to Mg was the effect of phosphate, which inhibited contraction with succinate much more than with ATP (fig 15). Investigation of this phenomenon revealed that addition of ADP in the presence of a hexokinase trap would partially reverse the phosphate inhibition (fig 16). Presumably, the inhibitory action of phosphate is through the oxidative phosphorylation mechanism and an acceptor system for the phosphate reverses the inhibition. It must be emphasized that substrate alone is all that is required for contraction. The respiration does not have to be coupled to phosphorylation as with many animal preparations (21, 26). In other experiments, it was found that the effects of succinate and ATP are not additive; both lead to the same level of contraction, the succinate very rapidly. The phosphate inhibition was studied with Mg and oligomycin as variables (fig 17). The inhibition was not materially altered, indicating that the entry of phosphate into the sensitive site does not require exogenous Mg, nor is it blocked by oligomycin.

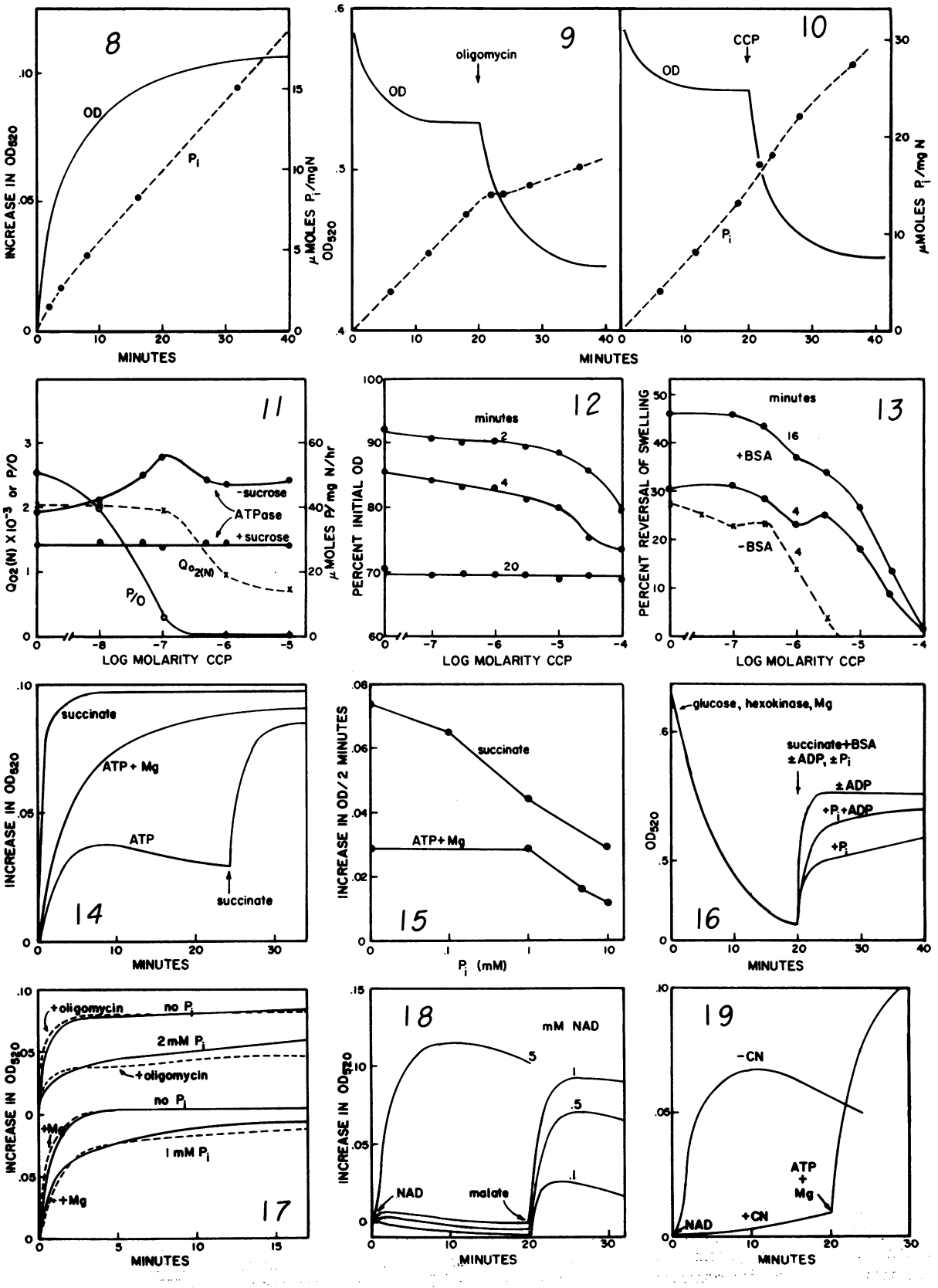
Contraction was studied with other substrates. Malate serves as well as succinate, particularly if NAD is added. Pyruvate was ineffective alone, but quite active if a malate or succinate "sparker" was added. Very rapid contraction was obtained with NADH (1 mM).

One oddity appeared in studying contraction with NAD, used as a control for NADH. While 1 mM NAD would not give contraction (1 mM NADH would), 5 mM NAD gave good contraction, although there was a slight delay (fig 18). The subsequent addition of malate with low levels of NAD caused a rapid contraction to a level conditioned by the amount of NAD added. The contraction caused by 5 mM NAD could be completely blocked by cyanide (fig 19). Cyanide did not block ATP-powered contraction. It was decided that the high concentration of NAD probably activated the oxidation of endogenous substrate, producing the noted contraction. Presumably, the corn mitochondria we work with are sufficiently depleted of NAD that endogenous substrate oxidation of this type is of little consequence in the other experiments we report here.

Cyanide was also effective in blocking contraction with other substrates. Furthermore, if mitochondria were held contracted with succinate, the addition of cyanide permitted rapid swelling (fig 20). Note also in figure 20 that addition of Pi causes the rapid establishment of a new, lower equilibrium.

Table III lists the percentage inhibition of contraction obtained with other inhibitors of electron transport. Rotenone was ineffective with succinate but effective with malate, which is in accord with its specific site of action in blocking NADH oxidation (5). The specificity of rotenone was verified in the Warburg apparatus: At 0.01 mM rotenone, the oxidation rate of malate + pyruvate decreased [e.g., Qo_2 (N) from 1470–650], while the oxidation of succinate alone increased (740–1200). No investigation was made of this respiratory increase, but it was not due to uncoupling as the P/O values were not significantly altered. The response may be related to the observation that succinate is poorly oxidized by these corn mitochondria unless pyruvate is added (9), presumably due to accumulation of inhibitory concentrations of oxalacetate (25). Rotenone would be expected to inhibit oxalacetate production by virtue of its ability to block oxidation of NADH produced during the conversion of malate to oxalacetate.

Oligomycin was ineffective with substrate-powered contraction in contrast to its effectiveness with ATP-powered contraction (table III).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INCREASE IN OD820
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11
QO2(N) X10-3 or P/O
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glucose, hexokinase, Mg
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±ADP, ±Pi
±ADP
+Pi+ADP
+Pi
OD520
MINUTES
17
+oligomycin
no Pi
2mM Pi
+oligomycin
no Pi
+Mg
1mM Pi
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INCREASE IN OD520
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Table III. *Inhibition of Substrate-Powered Contraction*

Percentage inhibition calculated from change in OD 4 minutes after adding substrate. Ethanol was added to control where used with inhibitor.

Substrate	Inhibitor		% Inhibition
Succinate	Antimycin	0.01 mM	100
Succinate	Malonate	8 mM	93
Malate	Malonate	8 mM	0
Succinate	Amytal	5 mM	51
Malate	Amytal	5 mM	91
Succinate	Rotenone	0.01 mM	7
Malate	Rotenone	0.01 mM	61
Succinate	Oligomycin	1 μg/ml	0
ATP + Mg	Oligomycin	1 μg/ml	83

The action of CCP was again most peculiar. At concentrations which uncouple the mitochondria without affecting respiration (fig 7), there is an increase in contraction rate (fig 21). Beyond 10^{-6} M CCP, however, there is a strong inhibition. Figure 21 also shows that BSA acts to increase the concentration of CCP needed to produce a response. Since the shape of the curves is not changed, it is likely that BSA binds CCP, reducing the effective concentration.

Substrate (succinate or pyruvate + succinate) added initially was just as effective as ATP + Mg in preventing swelling (fig 20). However, this was only true at pH 7.0 to 7.5 (fig 22). With higher pH, contraction could not be maintained, and at pH 8.5 swelling was almost as rapid as if no substrate had been added. We have not yet investigated this phenomenon in detail, but it would appear most important for understanding substrate-powered contraction. ATP-powered contraction was not studied above pH 8.0, but the results reported in figures 5 and 6 suggest that a similar response could be expected.

Swelling in Sucrose. The failure to obtain swelling in sucrose was of interest, and some experiments were conducted to see if swelling could be initiated. It was learned that if mitochondria were allowed to age in sucrose at room temperature, the addition of succinate would bring about rapid swelling. At first it was thought that this response was related to the respiration-dependent swelling in animal mitochondria, but further work revealed that the swelling was insensitive to antimycin A, and that a variety of dicarboxylic acids or phosphate would produce the same result. Citrate and EDTA were particularly effective, presumably because they are effective chelating agents. A time-course study with citrate is shown in figure 23. The degree of swelling is proportional to the time of aging in the cuvette. When citrate is added initially, no swelling is produced. We conclude that the stability of mitochondria in sucrose is dependent on some cation-stabilized membrane structure. With aging this structure weakens to the point where chelating agents can remove the cations, the organization of the structure is destroyed, and permeability is greatly increased. It is possible that this time-of-addition effect of citrate is through the type of lipid peroxidation studied by Hunter et al. (11). Lynn et al. (20) have recently shown that the permeability of liver mitochondria membranes can be strikingly altered by small amounts of Ca or fatty acids.

FIG. 8. The release of Pi accompanying ATP induced contraction. Contraction initiated with 3 mM ATP, 2 mM $MgCl_2$ and 1 mg/ml BSA. Aliquots withdrawn at different times for Pi analysis.

FIG. 9, 10. The release of ATP-maintained contraction by oligomycin and CCP, and the resultant effect on adenosine triphosphatase. Concentrations: 3 mM ATP, 2 mM $MgCl_2$, 1 mg/ml BSA initially; 2 μg/ml oligomycin and 10^{-5} M CCP added.

FIG. 11. The effect of CCP on oxidative phosphorylation and adenosine triphosphatase. The adenosine triphosphatase studies were made with a separate lot of mitochondria in 0.2 M KCl + 0.02 M Tris (pH 7.5) ± 0.5 M sucrose.

FIG. 12. Swelling rate of corn mitochondria as a function of CCP concentration.

FIG. 13. ATP-induced contraction of corn mitochondria as a function of CCP concentration. The CCP was added initially, and contraction induced at 20 minutes with 3 mM ATP + 2 mM $MgCl_2$ ± 1 mg/ml BSA.

FIG. 14. The Mg requirement for ATP induced contraction, and the rapid contraction produced by succinate alone. Mitochondria were allowed to swell for 20 minutes in the KCl-Tris medium (not shown), then contracted with 1 mM ATP + 1 mg/ml BSA ± 1 mM $MgCl_2$ or with 8 mM succinate + 1 mg/ml BSA.

FIG. 15. The inhibition of succinate- and ATP-powered contraction by phosphate. Contraction initiated after 20 minutes swelling in the KCl-Tris medium with 8 mM succinate or 3 mM ATP + 2 mM $MgCl_2$, both with 1 mg/ml BSA.

FIG. 16. The partial relief of phosphate inhibition of succinate-powered contraction by ADP acceptor plus a hexokinase trap. Concentrations: glucose, 5 mM; hexokinase 5 KM units/ml; $MgCl_2$, 1 mM; succinate, 8 mM; BSA, 1 mg/ml; ADP, 1 mM; Pi, 5 mM.

FIG. 17. The failure of Mg and oligomycin to alter Pi inhibition of succinate-powered contraction. After 20 minutes swelling in the KCl-Tris medium contraction was initiated with 8 mM succinate + 1 mg/ml BSA. Concentrations of additives: 1 mM $MgCl_2$; 1 μg/ml oligomycin (added initially); 2 mM (upper experiment) and 1 mM (lower experiment) potassium phosphate, pH 7.5.

FIG. 18. Contraction obtained with various concentrations of NAD, and the enhanced contraction resulting from subsequent addition of 8 mM malate.

FIG. 19. Inhibition of NAD contraction by cyanide, and the ineffectiveness of cyanide in preventing ATP-powered contraction. Concentrations: NAD, 5 mM; KCN, 5 mM; ATP, 3 mM; $MgCl_2$, 2 mM; 1 mg/ml BSA was added with the NAD.

They visualize respiration to result in an exchange of H^+ for Ca^{++}, releasing Ca^{++} to react with acidic phospholipids.

Discussion

Corn mitochondria clearly show a spontaneous swelling in KCl, but not sucrose. Only after aging and treatment with a chelating agent can the mitochondria swell in sucrose. The swelling is assumed to be accompanied by penetration of the suspending solute, KCl in the experiments to be discussed here. Spontaneous swelling must be differentiated from the rapid osmotic adjustment, which we have minimized by using media isoosmotic with the isolation medium. Spontaneous swelling is slow and appears to reflect the osmotic adjustment which follows solute penetration and the relaxation of some contractile mechanism. Water enters with relaxation and is expelled on re-establishment of the contracted state (fig 2), a process which requires energy from hydrolysis of ATP or oxidation of substrate. Spontaneous swelling is not in itself deleterious to subsequent oxidative phosphorylation, although there does appear to be some inhibition of electron transport if swelling occurs in the absence of BSA (table I).

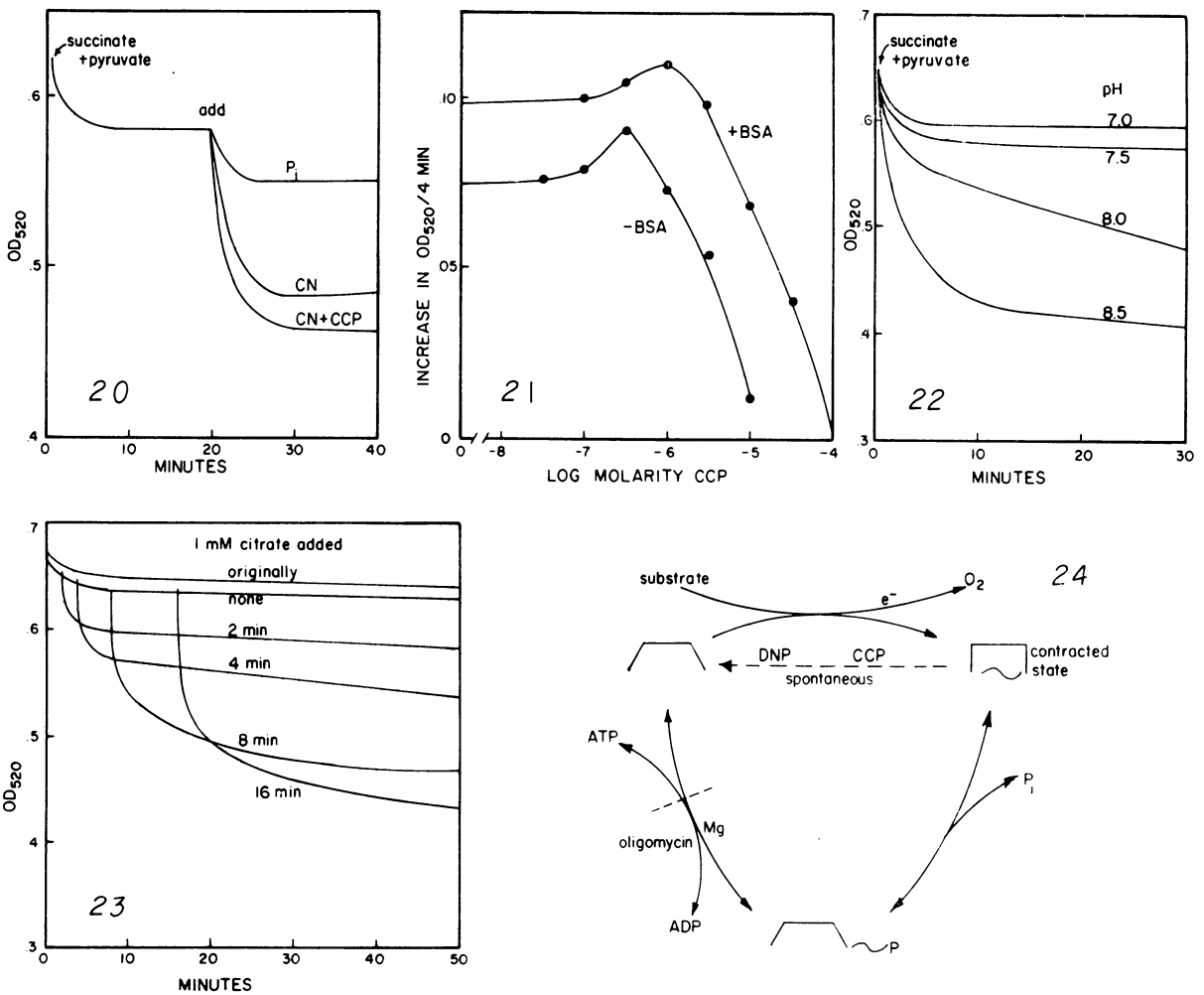

FIG. 20. Induction of swelling by phosphate and cyanide, and the additional swelling induced by the uncoupler CCP in cyanide inhibited mitochondria. Mitochondria maintained in contracted state with 8 mM each of succinate and pyruvate; swelling induced with 5 mM potassium phosphate or 1 mM KCN ± 10^{-6} M CCP.

FIG. 21. The effect of CCP on contraction initiated by 8 mM succinate ± 1 mg/ml BSA. Previous swelling was for 20 minutes in the KCl-Tris medium.

FIG. 22. The inability of succinate-pyruvate (8 mM each) to maintain contraction above pH 7.5. Medium was 0.2 M KCl + 0.02 Tris-maleate buffer with the pH adjusted as indicated.

FIG. 23. Citrate-induced swelling of mitochondria maintained in the unswollen state with 0.4 M sucrose. Citrate (1 mM adjusted to pH 7.5 with KOH) added at time intervals indicated.

FIG. 24. A scheme to explain the relationship between oxidative phosphorylation, adenosine triphosphatase, swelling and contraction.

The electron micrographs indicate that the mechanism is associated with the inner membrane. Micrographs of liver mitochondria also show the changes to be associated with the inner membrane and matrix (20).

There are 2 principal distinctions to be made between the behaviour of corn mitochondria and most animal preparations: No swelling agents are needed, and contraction can be obtained with substrate alone (i.e., nonphosphorylating respiration).

With respect to spontaneous swelling, we can only suggest that the corn mitochondria must be very permeable compared with most animal preparations. For instance, corn mitochondria oxidize exogenous DPNH very rapidly (data to be published separately) which is a characteristic of many plant preparations (8) but not of tightly coupled animal mitochondria (16).

The only report we know concerning nonphosphorylating substrate-induced contraction in animal mitochondria is the recent work of Lynn et al. (20). In this case, rat liver mitochondria were allowed to swell in succinate. Upon the addition of EDTA, there was a rapid contraction which was antimycin-sensitive, oligomycin-insensitive. These are similar to the results we have obtained, and it is of interest that our isolation and washing media include EDTA. The differences exhibited by corn and liver mitochondria may be a reflection of isolation procedures, rather than of intrinsic properties.

It is generally realized that there must be some connection between the high energy intermediate system of oxidative phosphorylation and contraction. A schematic representation of the relationship as we deduce it to occur in corn mitochondria is shown in figure 24. It is a modified type I scheme which we have used and explained previously (9, 29). The scheme makes no attempt to depict how high energy intermediate formation is linked to electron transfer.

The scheme attributes contraction to a mechanism associated with the nonphosphorylated high energy intermediate. It is assumed that the degree of contraction is a function of the relative amount of intermediate in this state. In the absence of substrate or ATP, spontaneous hydrolysis of the intermediate results in swelling. The addition of phosphate accelerates the breakdown of the intermediate by phosphorolysis. The oxidation of substrate directly reestablishes the intermediate (and expulsion of water follows, presumably at a slower rate), but in the presence of phosphate there is a rapid conversion to the phosphorylated intermediate. In the presence of a trap, the phosphate is transferred to ADP, regenerating the intermediate for recoupling. This accounts for the sparing-action of a trapping system on the phosphate inhibition of contraction (fig 16).

The production of the nonphosphorylated high energy intermediate from ATP is indicated to be accompanied by a stoichiometric yield of phosphate. The inhibitory effects of added phosphate on ATP-powered contraction (fig 15) could be attributed to mass action. The site of oligomycin action in blocking ATP-powered contraction and adenosine triphosphatase is the same as that depicted by Packer et al. (24) and Lardy et al (14); i.e., in the transfer of phosphate between ATP and the phosphorylated intermediate. Oligomycin cannot act in preventing phosphorylation of the intermediate, as is thought to be true for liver mitochondria (28), for it does not prevent the phosphate inhibition of contraction (fig 17). The respiration in the presence of oligomycin (table II) can be accounted for by assuming that there is an accelerated spontaneous hydrolysis of the nonphosphorylated high energy intermediate when the normal ADP-acceptor system is blocked: this short circuit would recycle the intermediate providing for some electron flow.

The continued release of Pi from ATP after contraction is accomplished is assumed to result from continued spontaneous hydrolysis of the nonphosphorylated intermediate (fig 8, 24).

There is one set of observations which is not readily explained by the scheme. The uncoupling action of CCP (or DNP) is generally considered to be in the hydrolysis of the nonphosphorylated high energy intermediate (34), which would account for the CCP-stimulated adenosine triphosphatase (fig 11). If this intermediate is closely linked to the contracted state, acceleration of swelling with CCP should be obtained with the uncoupling concentrations of 10^{-7} M (fig 11). However, CCP concentrations 10 to 100-fold higher are needed to accelerate swelling (fig 12) and inhibit contraction (fig 13, 21). In a physiological sense, concentrations of uncoupler are needed which begin to suppress respiration. The significance of this is not clear to us, although a reasonable postulate would be that the inhibited respiration is a reflection of a loss of membrane semipermeability.

The oligomycin-inhibited, CCP-stimulated adenosine triphosphatase (table II and fig 11) must reflect the loss of Pi from ATP through the intermediate system. The fact that the CCP-stimulated adenosine triphosphatase is not found in the presence of high levels of sucrose could be due to a limitation by sucrose on the entry of ATP into the mitochondrial adenosine triphosphatase sites. This effect of sucrose was noted earlier by Johnson and Lardy (13) for several Krebs cycle acids, and has been confirmed in our laboratory.

At the present time, we can go no further in correlating and explaining the results. There is obviously some very important connection between high-energy intermediates and membrane function, but it will not be resolved until the biochemistry of the intermediates is described. Correlative studies are also needed on ion transport accompanying swelling and contraction, for it is by no means certain that the squiggle used to designate the nonphosphorylated high energy intermediate (fig 24) really represents a covalent bond. The charge separation theory of Mitchell (22) could be invoked to explain contraction as

associated with removal of H^+ and OH^- ions from a hydrophobic adenosine triphosphatase center.

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